MEMORANDUM

SUBJECT: Human Health Hazard Assessment for TERA R19-01 – **NOT CBI**

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SUMMARY

There is low concern for human health effects resulting from the intended use of the intergeneric *Parachlorella* sp. There is also low concern for pathogenicity to the susceptible subpopulation of immunocompromised individuals. There is low concern for toxicity to humans as *Parachlorella* sp is not known to produce any toxins. There is low concern for allergenicity to the general population.

I. INTRODUCTION

EPA has received a TSCA Environmental Release Application (TERA) from Synthetic Genomics, Inc. to test one intergeneric eukaryotic algal strain in open ponds. The subject microorganism of this non-CBI TERA R-19-0001 is the green alga *Parachlorella* sp., strain STR26155. The wild-type parent strain STR00010 strain was obtained off the coast of the Hawaiian island of Oahu. The recipient strain STR00012, which was derived from STR00010 following UV mutagenesis, was engineered to express a "Turbo" green fluorescent protein (GFP) gene. The TurboGFP gene was obtained from Evrogen (Evrogen Joint Stock Company, Moscow, Russia, Catalog #FP511). TurboGFP was developed by Evdokimov et al. (2006) by performing solubility-enhancing optimization of ppluGFP2, the Copepoda GPF originally isolated from the *Pontellina plumata* in the order Calanoida (Shagin et al., 2004). TurboGFP is a highly water soluble, rapidly maturing variant of ppluGFP2 with a brighter fluorescence than ppluGFP2. According to the Genetic Construction Report (Cameron, 2019) this specific TurboGFP gene purchased and used in creation of STR26155 has been codon-optimized for expression in mammalian cells by Evrogen according to the method used by Haas et al. (1996) for optimizing expression of proteins in mammalian systems.

The introduction of the TurboGFP into the recipient strain was done to enable environmental tracking of the subject strain STR21655. Environmental monitoring of the recipient strain STR00012 has been done for 18 months. The genetic engineering involved the use of CRISPR-Cas9, transformation by electroporation, and the use of the loxP-Cre recombinase system for removal of antibiotic resistance genes used in selection of intermediates. Although resistance genes for chloramphenicol and zeocin were used as selection markers, no antibiotic resistance genes remain in the subject microorganism STR26155.

The final strain STR26155 contains the TurboGFP gene, an intergeneric loxP site, and an intrageneric *HpaI* (restriction enzyme) site.

This is the fourth TERA received by the Agency for open pond testing of genetically engineered algae. Previous algae TERAs include [redacted], and R-18-0001.

II. GENETIC MODIFICATIONS

A. RECEPIENT MICROORGANISM

The subject microorganism of this non-CBI TERA R-19-0001 is the green alga *Parachlorella* sp., strain STR26155. The recipient strain STR00012, which was derived from STR00010 following UV mutagenesis, was engineered to express a "Turbo" green fluorescent protein (GFP) gene.

B. DONOR ORGANISMS

A plasmid (NAS14335) containing the TurboGFP cassette was constructed via Gibson cloning of eight linear DNA fragments, synthetic linkers, and genes, PCR-amplified endogenous regulators, and intermediate plasmids. This plasmid was digested with a restriction enzyme prior to co-transformation into the recipient using a CRISPR Cas9 nuclease ribonucleoprotein (RNP) complex (Cas9 with guide RNA). The plasmid backbone contained a chloramphenicol resistance gene, but this gene as well as several others dropped out of the intermediate strain as the backbone of the plasmid did not have the ability to self-replicate. Sequencing of the subject microorganism confirmed the backbone did not integrate off-target in the genome.

This NAS14335 plasmid was originally assembled so that the Cre recombinase and *ble* genes were in between the two *loxP* sites so that Cre recombinase could cause self-excision of the DNA between the *loxP* sites. The Cre recombinase was then induced in the presence of nitrate which resulted in the removal of the *ble* gene (zeocin resistance) used as a selection marker. The removal of the *ble* gene was verified by zeocin sensitivity.

The intergeneric TurboGFP gene and one *loxP* site (from bacteriophage P1) remains in the subject microorganism STR26155. There is also one intrageneric *HpaI* site remaining in STR26155 from the genetic modifications.

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III. HUMAN HEALTH HAZARDS OF THE RECEPIENT MICROORGANISM

Parachlorella is a genus in the family *Chlorellaceae* which is also comprised of the genus *Chlorella*. *Chlorella* has been found throughout all of North America from tropical to arctic climates. *Chlorella* spp. are omnipresent in both aquatic and terrestrial environments (Hodac et al., 2016). Like many other algae, *Chlorella* is an important primary producer and food source for higher trophic levels.

A. PATHOGENICITY

There is no evidence in the literature that the *Parachlorella* spp. causes infections in humans.

However, in extremely rare cases, *Chlorella* has caused infections in humans and other animals. As mentioned previously, since the genus *Parachlorella* was split out from *Chlorella*, it is likely that older reports and studies on *Chlorella* may also apply for the genus *Parachlorella*.

Chlorellosis is the name of a rare *Chlorella* infection that has occurred in limited numbers in sheep and cattle, and in single cases in a human, dog, gazelle, beaver, camel, and fish (as summarized by Hart et al., 2014). Animals are infected by exposure of open wounds to contaminated water. In mammals this disease ranges from localized cutaneous infection, lymph node infection, or dissemination to multiple organs. However, in humans, the three reported cases were cutaneous infections (Jones et al., 1983; Yu et al., 2009; Hart et al., 2014). Chlorellosis in humans is extremely rare as there have been just three reported cases when the alga *Chlorella* is prevalent globally in fresh water lakes and rivers, in marine waters, and in soil. Another green alga, *Prototheca* that has been shown to infect humans at a higher rate (more than 100 cases have been reported). However, infection by *Prototheca* is also rare as this alga is widespread in the environment and thus, humans are highly exposed.

The first case of chlorellosis in humans was described by Jones et al. (1983) where a 30-year-old woman developed a persistent infection of a healing operative wound on her right foot after possible contamination by river water while canoeing. The wound was debrided two months later and the infection then treated with antibiotics and wound irrigation. The infection was persistent and healed completely after 10 months.

The second case of *Chlorella* infection was an external infection found in the gangrene tissue from the right foot of a diabetic 59-year-old female (Yu et al., 2009). The *Chlorella* isolate was thought to be *C. saccharophila,* a *Chlorella* strain that uses glucose as a sole carbon source, grows at pH 2-3, and grows at temperatures up to 30°C. The authors stated the strain "could not grow at 37°C in light or darkness. The results suggest that this strain may not normally invade tissues, but becomes established and grows on previously infected tissues of external body extremities where the temperature is somewhat lower than normal body temperature."

The most recent case of chlorellosis was reported in Australia in a 30-year-old man in a knee wound contaminated with fresh water dam water (Hart et al., 2014). He developed a *Chlorella* and *Aeromonas hydrophila* infection within two days of exposure and the infection was aggressive and required debridement, negative pressure wound dressings, and antibiotics. However, the wound had healed by the third week with no further complications.

Overall, chlorellosis in humans is extremely rare as there have been just the three reported cases mentioned above, even when the alga *Chlorella* is known to be widespread. Even with more than 100 cases reported of human infections from the alga, *Prototheca*, it is also still considered rare since *Prototheca* is widespread in the environment, where humans are constantly exposed to. The fact that such few *Chlorella* infections have been reported, and considering that *Chlorella* is a prevalent alga in fresh water, marine waters, and in soils where humans are frequently exposed to the alga, implies that chlorellosis is quite rare.

B. TOXICITY

According to the submission, there are no reports in the literature that any *Parachlorella or Chlorella* species, synthesizes or secrets toxins.

The lack of toxin production by *Chlorella* allows it to be used as a popular human nutritional supplement. In addition, *Chlorella* extracts are used in skin care products. *Chlorella* has been

proposed as a protein supplement for human consumption (Becker, 2007). *Chlorella* sp. are generally regarded as safe (GRAS) for human consumption. *Chlorella* sp. and *C. protothecoides* flours have GRAS status (GRN 000330; GRN 000519) with the Food and Drug Administration (FDA). In humans, *Chlorella* sp. supplements have shown beneficial effects including improved immune responses, improved healing of the small intestine epithelium, antioxidant action and even anti-tumoral effects (Ramirez-Romero et al., 2010). *C. vulgaris* has been promoted as a prevention of anti-inflammatory responses (Hasegawa et al., 1999). Morin et al. (1980) have shown inhibitory effects of the unicellular alga *Chlorella* against murine sarcomas. Since *Parachlorella* had previously been classified as *Chlorella*, Buxser (2019) concluded that *Parachlorella* have been used as human food for many years already.

There is one study in the literature that reported cytotoxicity of algal dietary supplements consisting of a mixture of *Chlorella* sp. and the collective cell biomass from two cyanobacteria, *Arthrospira platensis* and *A. maxima* commonly referred to as *Spirulina* (Heussner et al., 2012). They found extracts from 13 commercially available products sold in Germany were cytotoxic in the A549 cell line with the *Spirulina* being more potent than *Chlorella*. This toxicity, however, was due to contamination of the cyanobacterial and algal cultures by microcystin, a potent toxin produced by the cyanobacterium *Microcystis.* The toxicity was not due to the *Chlorella* or *Spirulina.*
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Altogether the available evidence indicates a low concern for toxicity of *Chlorella* or *Parachlorella* based on its use as a food supplement and skin care product.

C. ALLERGENICITY

A search of "Parachlorella" AND "sensitization" OR "allergenicity" in PubMed did not result in any published references. As with the previous sections on pathogenicity and toxicity, studies and reports that have been done in the past on *Chlorella*, are very likely to be applicable for *Parachlorella*.

Allergy is the result of a marked increase in reactivity and responsiveness of an immune response to a protein or a low molecular weight compound combined with a larger "self" molecule. However, recent research suggests that not every protein is allergenic (Radauer et al., 2008).

Humans may be routinely exposed to high numbers of algal cells on a daily basis through respiration in both indoor and outdoor environments. Algae and cyanobacteria usually constitute a minority of airborne bioaerosols compared to fungi, pollen, and bacteria; however, in certain cases the quantity of airborne algal particles can far exceed that of fungi spores and pollen grains (McGovern et al., 1965). Brown et al. (1964) found over 3000 algae/m^3 in samples taken from a car moving through a dust cloud in Texas. Schlichting (1969) found < 8 algal cells/ft^3 in air sampled in Texas, Michigan, and off the North Carolina coast and calculated that breathing 240 algae cells per hour was possible for a maximum daily uptake of 2880 algal and cyanobacterial cells. In a summary of the existing literature on airborne algae during the years 1910 - 1968, a total of 187 taxa of algae and protozoa were found. Several species of *Chlorella* were sampled directly from the air including *C. ellipsoidea, C. pyrenoidosa, C. vulgaris,* and *Chlorella* sp. (Schlichting, 1969). Bernstein and Safferman (1970) also found 18 different genera of algae in house dust collected from 41 homes of which *Chlorella* was the most frequently encountered algae, followed by *Chlorococcum, Schizothrix, Planktosphaeria, Chlamydomonas, and Anabaena.*

There is evidence from human studies that *Chlorella* can induce hypersensitivity responses in some individuals. Tiberg et al. (1995) tested Swedish children for allergy to *Chlorella* using three methods: the radioallergosorbent test (RAST), skin prick tests (SPTs), and conjunctival provocation tests (CPT). These tests detect specific IgE antibodies to determine whether a subject is sensitized to the substance. No *Chlorella*-specific IgE antibodies were found in the sera from the 94 children from the general population (group 1 – no allergy symptoms). In a group of children that had been referred to an outpatient pediatric allergy clinic (group 2), nine of the 129 children had positive wheal reactions with the *Chlorella* extract in SPTs. Sera from seven of these children with positive SPTs results were available for analysis of IgE antibodies. Two of the seven were positive for IgE-specific antibodies to Chlorella. Seven of 23 mold-sensitive children (group 3) had positive SPTs to *Chlorella.* Six patients with SPT positive results and two of the 16 patients with negative SPT results had positive RAST results. All patients with positive SPT results showed some reaction in CPTs with *Chlorella* extract (5 mg dry weight/ml). These data demonstrate that only children that are sensitized to many common allergens also were sensitized to *Chlorella* and no specific symptoms related to *Chlorella* sensitization were observed. These data suggest that *Chlorella* is a weak allergen.

Similarly, Bernstein and Safferman (1966) tested two species of *Chlorella, C. vulgaris* and *C. pyrenoidosa,* two species of *Chlorococcum, C. botryoides* and *C. macrostigmatum*, *Scenedesmus basilensis,* and *Ankistrodesmus falcatus* var. *acicularis* for their potential to elicit cutaneous reactions in atopic patients, i.e., those with a genetic predisposition for developing allergic hypersensitivity reactions. They found that of 79 atopic patients tested with algal extracts, 47 also gave positive skin reactions while non-atopic individuals did not show positive skin reactions. Additional tests with *C. vulgaris* for bronchial mucosa tests resulted in clinical wheezing. Interpretation of this study is greatly limited by lack of antigen quantification or understanding of the cellular and molecular mechanisms involved and small sample sizes.

There is a single report of occupational asthma in a pharmacist induced by exposure to a fine dust powder of *Chlorella* while making chlorella tablets for human consumption (Ng et al., 1994). It was suggested that the causative agent in this chlorella-induced asthma was pheophorbide-a, which is a breakdown product of chlorophyll, and its ester, or some other protein component. Pheophorbide-a and its ester are formed by the reaction of the chlorophyllase enzyme during the drying process of the moist *Chlorella* cells with heated air at 90°C. Given that the hypersensitivity response was induced by fine, dry dust created by high heat, the relevance of this report of occupational asthma to exposures of live moist *Chlorella* cells in bioaerosols during this field test is questionable.

The database Allergome lists *Chlorella* as an allergen, however the WHO/IUIS Allergen Nomenclature database (Allergen Nomenclature (IUIS); http://www.allergen.org) does not. Based on a review of outdoor allergens, Burge and Rogers (2000) stated that algae do not seem to be a source of major outdoor allergens.

Chlorella has also been reported to cause photosensitization, which is development of abnormally heightened reactivity of skin or eyes to sunlight, in those who took *Chlorella* as a dietary supplement (Jitsukawa et al., 1984). In addition, protein components of *Chlorella* such as a breakdown product of chlorophyll, pheophorbide-a and its ester that are recognized as photosensitizers may contribute to adverse reaction in the kidney (Yim et al., 2007). However, this photosensitization resulted from ingestion of algae which is not relevant to exposures in this TERA field test with the closely-related *Parachlorella*.

IV. HUMAN HEALTH HAZARDS OF THE SUBMISSION MICROORGANISMS

The subject strain is engineered to express the TurboGFP for monitoring in the environment. As previously stated, TurboGFP is a variant of the ppluGFP2 originally isolated from the copepod *Pontellina plumata* (Shagin et al., 2004). This specific TurboGFP purchased from Evrogen is a version of the TurboGFP developed by Evdokimov et al. (2006) that has been codon-optimized by Evrogen for expression in mammalian cells following the method of Haas et al. (1996).

The concern for pathogenicity or toxicity associated with the introduced gene is low. As described by the submitters and the Construct Hazard Analysis (McClung, 2019), the introduced DNA coding for TurboGFP, is not expected to introduce any other phenotypic change in the recipient microorganism and does not impart or enhance any harmful traits beyond what may be present in the recipient strain.

There is one study in the literature assessing the toxicity of GFP fed to rats as pure protein and in a diet consisting of transgenic canola expressing GFP (Richards et al., 2003). The authors reported that oral administration of 1.0 mg of purified GFP/day for 26 days was not toxic to male rats. GFP is widely used in scientific applications and no reports were identified in PubMed database for GFP toxicity or allergenic effects.

The protein sequence encoded by the introduced GFP gene was used to query the Food Allergy Research and Resource Program "AllergenOnline". Database queries using the full 233 amino acid sequence, a sliding 80mer window, as well as 8mer did not idenfity any results above the database threshold indicating an extremely low likelihood of allergenicity for the encoded GFP protein.

LoxP is widely used in genetic biomedical research and is not known to pose any human health hazards.

Although resistance genes to chloramphenicol and zeocin were used in the development of the subject strain STR26155, they are not present in this final submission strain.

Altogether, no human health hazards were identified for the introduced sequences, TurboGFP and loxP.

V. **RISK TO POTENTIALLY EXPOSED AND SUSCEPTIBLE SUBPOPULATIONS**

Potentially exposed individuals are workers at the SGI facility. Susceptible subpopulations that warrant consideration differ whether in relation to potential pathogenicity or allergenicity of *Parachlorella.* In terms of pathogenicity, susceptible subpopulations would include those whose immune systems are not fully competent such as the young, the elderly, malnourished individuals, and those with pre-existing disease or on immunosuppressive therapies. Susceptible populations for allergenicity concerns are atopic individuals which are those with a genetic predisposition toward developing hypersensitivity reactions to environmental antigens.

Parachlorella has not been reported as causing any infections in humans. However, there are three reports of *Chlorella* sp. infections in humans originating in open wounds after exposure to contaminated water. Chlorellosis is extremely rare even though humans are routinely exposed to

Chlorella as it ubiquitous in the environment in fresh waters, marine waters, and in soils, and even found in the indoor environment in house dust. Thus, there is little concern even for those with not fully competent immune systems as they too are routinely exposed to *Parachlorella* sp. Dermal contact of workers to the alga in the open miniponds is not expected as workers will be wearing personal protective equipment required by SGI regulations (e.g., gloves, safety glasses, long pants, and steel-toed shoes) when handling the algae.

In regards to toxicity, there is low concern for potentially exposed or susceptible subpopulations as well as the general population as *Parachlorella* is not known to produce any phycotoxins.

There may be some concern for allergenicity with potentially exposed and susceptible subpopulations if any workers are atopic individuals that are prone to developing hypersensitivity reactions even though *Chlorella* has been characterized as being a "weak" allergen (Tiberg, 1995). Bioaerosols containing algal cells are expected to be generated during the growth of the algae in open raceway miniponds so some inhalation of the submission strain *Parachlorella* sp. STR00012 is expected. The general human population does not appear to suffer allergenicity symptoms from exposure to *Chlorella* since *Chlorella* is ubiquitous in the environment in fresh water, marine waters, and soils, and even occurs in house dust so humans routinely inhale *Chlorella* cells. Based on a review of outdoor allergens, algae do not seem to be a source of major outdoor allergens (Burge and Rogers, 2000). It is unlikely that atopic individuals would choose to work with algae given their predisposition to developing hypersensitivity reactions. However, if atopic individuals work at the facility, allergenicity symptoms could be alleviated by the use of respirators (APF50 respirators with P100 filters that removes 99.97% of exposure to microorganisms).
Considering the widespread existence of algae species and the extreme rarity of algae infections, it is unlikely that *Parachlorella* presents a health hazard to susceptible subpopulations. No other hazard concerns were identified for the recipient or donor organisms.

VI. **CONCLUSIONS**

Overall, there is low human health concerns for the submission microorganism.
The genetic modifications of the recipient to make the submission strain *Parachlorella* sp. STR26155 strain do not pose adverse human health effects to potentially exposed and susceptible subpopulations just as they do not to the general human population. The introduced TurboGFP does not pose pathogenicity, toxicity or allergenicity concerns to humans.

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